PCT / IB 98 / 01919
29. 12. 98
09/554704

The Patent Office

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

5

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated 30 NOV 1998

Patents Form 1/77

Patents Act 1977
(Ru[redacted]6)

The Patent Office

1/77

9
25NOV97 E319562-1 D01874
P01/7700 25.00 - 9724725.8

Request for grant of a patent

(See notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

CG/SAR/97125GB

2. Patent application number
(The Patent Office will fill in this part)

21 NOV 1997

9724725.8

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

SEE ATTACHED SHEET

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

PROTEIN PRODUCTION PROCESS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

BATCHELLOR KIRK & CO
2 PEAR TREE COURT
FARRINGDON ROAD
LONDON EC1R 0DS

Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

NO

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	1
Description	9
Claim(s)	2
Abstract	1
Drawing(s)	4

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent ~~on the~~ basis of this application.

Signature [signature] Date 21/11/97

12. Name and daytime telephone number of person to contact in the United Kingdom

CAROL GREAVES - 01934 844419

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

CONTINUATION SHEET FOR PATENTS FORM NO. 1/77

Full name, address and postcode of the or each applicant:

Catharina Svanborg

66 St John Wood High Street
London NN8 7SH
England

7328552001

Malin Wilhelmina Svensson

Kämnärs vägen 3B-126
S-226 46 Lund
Sweden

7328560001

Per Anders Håkansson

Flormans gatan 2A
s-223 54 Lund
Sweden

7328594001

Protein Production Process

The present invention relates to a process for the preparation of a therapeutic protein complex, and to apparatus and reagents for use in the process.

A multimeric form of α-lactalbumin or MAL has previously been reported as having therapeutic applications both in the field of antibiotic (WO96/04929) and cancer therapy (A. Håkansson et al., Proc. Natl. Acad. Sci USA, (1995) 92, 8064-8068).

MAL was obtained by passing a casein fraction of milk, particularly human milk, down an ion exchange column, specifically a DEAE-Tris-acyl M column using an NaCl gradient. A pool containing MAL which was active therapeutically was obtained. In addition, the conversion of commercially available monomeric α-lactalbumin to the multimeric form was reported.

The applicants have found an improved way of producing MAL in greater yields and from a greater variety of α-lactalbumin sources.

The present invention provides a method for producing a multimeric form of α-lactalbumin which comprises exposing a source of α-lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof and recovering α-lactalbumin in a multimeric form therefrom.

Pre-treatment or "training" the ion exchange medium with casein or active components thereof has been found to be particularly effective in increasing the yield of MAL, for

example from various sources such as commercially available α-lactalbumin derived from both human and bovine milk.

The expression "active component" used herein refers to those one or more elements found in casein which produce the desired improvement in the process when used as a pre-treatment of the ion exchange column. It is known for instance, that casein contains a number of lipids and occasionally free fatty acids. The fatty acid content of casein is marked increased if for example, the casein if frozen or subjected to hydrolysis reactions. It is possible that these components are retained on the column and give rise to the improved MAL production capability observed.

Casein from human milk contains polyunsaturated fatty acids like oleic acid, linoleic, γ-linoleic and arachidonic acid as well as triglycerides and lipids particularly phospholipids such as sphingomyelin. Any of these either alone or in combination, may produced similar enhancement effects on the column.

It would be a matter of routine to test which of these components or combination of components produced enhanced effects and then the reagents could be used alone in place of the casein in order to pre-treat the ion exchange material.

The casein or active component used in the pre-treatment step may be isolated from milk derived from various mammals, such as humans, bovines, sheep or goats. Preferably however the casein or active component used in the pre-treatment are derived from human milk.

Isolation of casein fractions can be carried out using known methods, for example as described in WO 96/04929. The casein may be used directly or it may be frozen and later thawed prior to use. It has been found that casein which has been frozen or derived from frozen human milk is preferred in the pre-treatment step. Where the casein is used directly, it is

preferable that it is first subjected to hydrolysis so as to hydrolyse some triglycerides present and so increase the amount of free fatty acid present before use in the pre-treatment step. This hydrolysis may be achieved for example by exposure of the casein to bile salts (J. Bitman et al., J. Ped. Gast. Nutr. 1983: 521-524).

Preferably the ion-exchange material used is arranged in a column as is conventional in the art. The various treatments can then be eluted through the column.

Suitably casein or the active components thereof are eluted through a column containing new unused ion exchange material such as DEAE Trisacryl. Suitable elution buffers include Tris-HCl with a pH of 8.5.

The amount of casein or active components applied to the column in this way may be small depending upon the volume of α-lactalbumin is required to be converted to MAL. For example, it has been found that only 30mg of casein or casein equivalents per ml of column material can be used in the conversion of multiple 10mg runs of bovine α-lactalbumin. After 6 runs (60mg), the yield began to decrease, but some multimeric α-lactalbumin was still obtained even after 10 runs.

In a preferred embodiment, the column is then washed with ion exchange buffer, such as the Tris-HCl buffer mentioned above, without casein, and preferably also other buffers which are to be used in the process, such as a NaCl containing buffer to ensure that nothing unspecific will elute from the column when used in the process. Washing may be done several times.

The column may then be eluted with the source of α-lactalbumin as described above dissolved in the ion exchange buffer. The column comprises anion exchange material. Suitably a salt concentration gradient is induced in the column by elution

with buffer containing suitable salts, for example those containing a suitable anion such as chloride. One such salt is sodium chloride. MAL containing fractions can then be isolated from the column. These fractions may be identified spectroscopically for example as illustrated hereinafter.

In the experiments illustrated hereinafter, it has been found that in general, the most active MAL fraction elutes first although some may be carried over into a second elution peak.

Using the process of the invention, MAL can be obtained from a wide variety of α-lactalbumin sources. For example it may be isolated from casein fractions or whey fractions of milk from any of the above-mentioned mammals or from commercially available monomeric α-lactalbumin which has been derived from any of these mammals. For example, monomeric bovine α-lactalbumin can be converted to therapeutic MAL in good yields, in some cases substantially completely, by treatment in accordance with the invention.

In some instances, depending upon the purification process used in its production, it may be preferable to contact the α-lactalbumin with a calcium chelating agent such as EDTA (ethylene diamine tetraacetic acid) in order to remove excess calcium. This may be applied as a pre-treatment in which the α-lactalbumin is contacted with the chelating agent prior to elution down the ion exchange column, or alternatively, the EDTA may be added to the elution buffer. Whether or not this is necessary in order to obtain optimum yields of active MAL can be determined by carrying out trial runs as illustrated hereinafter.

A pre-treated column can be used repeatedly to convert numerous fractions of α-lactalbumin to MAL. Once the column is exhausted or the conversion rate drops to unacceptable levels, the pre-treatment step can be repeated in order to restore the enhanced MAL production activity.

The reason why the "training" of the column in this way is so advantageous in MAL production is not entirely clear. It is possible that one or more components of the casein are retained on the column and thereafter catalyse MAL production or stabilise α-lactalbumin in its multimeric form.

Ion-exchange media and columns which have been trained or conditioned in this way form a further aspect of the invention, as does multimeric α-lactalbumin obtainable using this method.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows an ion exchange chromatogram obtained by elution of a sample of pre-treated human monomeric α-lactalbumin down a clean ion exchange column:

Figure 2 shows an ion exchange chromatogram obtained by elution of a sample of pre-treated-human monomeric α-lactalbumin and lipids down a clean ion exchange column:

Figure 3 shows an ion exchange chromatogram obtained by elution of a sample of human monomeric α-lactalbumin down a trained ion exchange column in accordance with the invention: and

Figure 4 shows an ion exchange chromatogram obtained by elution of a sample of bovine monomeric α-lactalbumin down a trained ion exchange column in accordance with the invention.

In the Figures, the protein peak is indicated by a thick line.

The ion exchange matrix used in the following examples was DEAE Trisacryl M from BioSepra, France. The buffers

comprised: Buffer A, 10mM Tris-HCl pH 8.5 and Buffer B, 10 mM Tris-HCl with 1M NaCl pH 8.5.

The experiments were carried out at room temperature (22°). Sample was dissolved in 10ml of buffer A.

Sample solution were injected onto the column at 1 ml/minute. The column was then eluted with buffer A for 10 minutes in order to get the sample on the column. Then a gradient of buffer B from 15-30% was induced. The gradient was held at 30% buffer B for 20 minutes during which time unwanted protein was eluted from the column. The concentration of buffer B was then increased to 100% which was held for 10 minutes. Where present, a MAL containing fraction eluted from the column during this period. The concentration of buffer B was reduced to 0% which was held for 20 minutes. Thereafter the concentration of buffer B was raised again to 100% and held for 20 minutes, during which a second MAL containing fraction was eluted. The concentration of buffer B was then reduced to 0% and the column eluted with buffer A only for a further 50 minutes.

Product was analysed using gel electrophoresis (Tris-Glycine PAGE gels 4-20%) as described previously in WO 96/04929, the content of which is hereby incorporated by reference.

Comparative Example A

This example was carried out using a new and previously unused ion exchange matrix. Monomeric α-lactalbumin (ca. 25mg) was added to the column in the manner outlined above. No multimeric peaks were obtained on this occasion.

Monomeric α-lactalbumin (20mg) was subjected to a procedure used in the precipitation of casein. Specifically, the sample was mixed with 10% potassium oxalate and incubated overnight at 4°C. The pH was then lowered to 4.3 and the sample incubated at 32°C for 2 hours. After o.n. incubation at

4°C, the sample was added to the column. Although small multimeric peaks were detected (Figure 1), no protein was found after dialysis and freeze drying of the product.

Monomeric α-lactalbumin (20mg) was mixed with lipids extracted from whole human milk, subjected to the casein precipitation procedure outlined above, and added to the column. Again small multimeric peaks were detected but no protein was found after dialysis and freeze drying (Figure 2).

Example 1

In this example, 300mg of casein derived from human milk was run on a fresh unused ion exchange matrix. The matrix was then washed with two runs of buffer A. Untreated monomeric human α-lactalbumin (8mg) was added to the column. Two multimeric peaks were found (Figure 3). Four further samples were run down this column and all gave two multimeric peaks.

Comparative Example B

Example A above was repeated using a different sample of unused matrix and with monomeric bovine α-lactalbumin samples. None of the samples gave any multimeric peaks.

Example 2

Example 1 above was repeated using monomeric bovine α-lactalbumin in place of human α-lactalbumin. Two clear multimeric peaks were obtained (Figure 4).

Example 3

Supplies of α-lactalbumin were obtained from various sources including commerically available human and bovine α-lactalbumin (Sigma) and α-lactalbumin isolated from human milk by ammonium sulphate precipitation of all proteins except α-lactalbumin, and then running the supernatant on a phenyl-sepharose column.

The samples were eluted down a column as described in Example 1. A second group of samples were pre-treated with EDTA by adding 7mg of the α-lactalbumin to 10ml of Buffer A containing 1mM of EDTA. The mixture was left at room temperature for about 3 hours and was then added to the trained column and run using the buffers set out in Example 1.

A third group of samples were treated with an EDTA containing buffer as described above and then run using EDTA buffers.

The two peaks which are believed to contain MAL were kept separately and tested individually. Representative results are set out in Table 1.

Table 1

Source of α-lactalbumin	EDTA addition	MAL Peak tested	Dosage	Viability of tumour cells*
Commercial human α-lactalbumin	Untreated	1	0.5mg/ml	0%
			1.0mg/ml	0%
		2	1.0mg/ml	0%
Human α-lactalbumin +	Untreated	1	1.0mg/ml	79%
		2	1.0mg/ml	95%
	EDTA pre-treated	1	1.0mg/ml	0%
		2	ca.1.5mg/ml	98%
	EDTA pre-treatment + EDTA buffer	1	0.5mg/ml	0%
			1.0mg/ml	0%
		2	0.5mg/ml	89%
			1.0mg/ml	0%
Commercial bovine α-lactalbumin	untreated	1	0.3mg/ml	50%
			0.5mg/ml	0%
		2	0.5mg/ml	98%
			1.0mg/ml	98%

* Cell viability of tumour cells incubated with MAL as described below.

+ Obtained using ammonium sulphate precipitation step as discussed above.

These results indicate, that the first MAL containing fraction to elute is the most active. It is possible that the second peak contains only some residual MAL which 'leaked' from the first peak.

With certain samples, and in particular, those isolated from human milk using an ammonium sulphate precipitation step as outlined above, the removal of some calcium is necessary in order to ensure efficient conversion of monomeric α-lactalbumin to MAL.

Biological Data

The biological efficacy of the products obtained in the above Examples was tested using tumour cell lines as described in . Cell lines were incubated with samples of the products at various concentrations in cell culture media at 37°C for various time points. After incubation, the cells are harvested and the cell viability determined using a vital dye (trypan blue). DNA was then prepared from the cells which was subjected to agarose gel electrophoresis. Analysis of the results revealed the occurrence of DNA fragmentation in all cases, indicative of apoptosis.

The activity of the products of the above Examples 1 and 2 was similar to that obtained using protein isolated from human milk casein as described by Håkansson et al (1995) supra.

Claims

1. A method for producing a multimeric form of α-lactalbumin which comprises exposing a source of α-lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof and recovering α-lactalbumin in a multimeric form therefrom.

2. A method according to claim 1 wherein the ion exchange medium has been treated with casein derived from human milk.

3. A method according to claim 1 or claim 2 wherein the ion exchange medium has been treated with casein which has been previously frozen or is derived from frozen milk.

4. A method according to claim 1 or claim 2 wherein the casein used in the pre-treatment of the ion exchange medium has been subjected to hydrolysis.

5. A method according to any one of the preceding claims wherein the ion exchange medium is arranged in a column.

6. A method according to any one of the preceding claims wherein the ion exchange medium comprise DEAE Trisacryl.

7. A method according to any one of the preceding claims which comprises eluting an ion exchange column with casein or the active components thereof in an ion exchange buffer, washing the column with ion exchange buffer, and then eluting the column with a source of α-lactalbumin dissolved in the ion exchange buffer in the presence of a salt concentration gradient.

8. A method according to claim 7 wherein the ion exchange buffer is Tris-HCl.

9. A method according to claim 7 or claim 8 wherein the salt concentration gradient is produced using an ion exchange buffer in which sodium chloride is dissolved.

10. A method according to claim 8 wherein the column is washed by elution of ion exchange buffer twice.

11. A method according to any one of the preceding claims wherein the said source of α-lactalbumin comprises monomeric bovine α-lactalbumin.

12. A method according to any one of the preceding claims wherein the said source of α-lactalbumin comprises monomeric human α-lactalbumin.

13. An ion exchange medium for use in the method of any one of the preceding claims, said medium having been treated with casein or an active component thereof.

14. An ion exchange column which comprises ion exchange medium according to claim 13.

15. A multimeric form of α-lactalbumin obtained by a method according to any one of claims 1 to 12.

Abstract

A method for producing a multimeric form of α-lactalbumin which comprises exposing a source of α-lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof, and recovering α-lactalbumin in a multimeric form therefrom. Pre-treatment of the ion exchange medium, particularly with casein derived from human milk, has been found to significantly improve yields of the multimeric form of α-lactalbumin and mean that it can readily isolated from readily available sources such as bovine α-lactalbumin.

This form of α-lactalbumin is useful therapeutically, in particular as an antibacterial agent and also as an anti-cancer therapeutic.

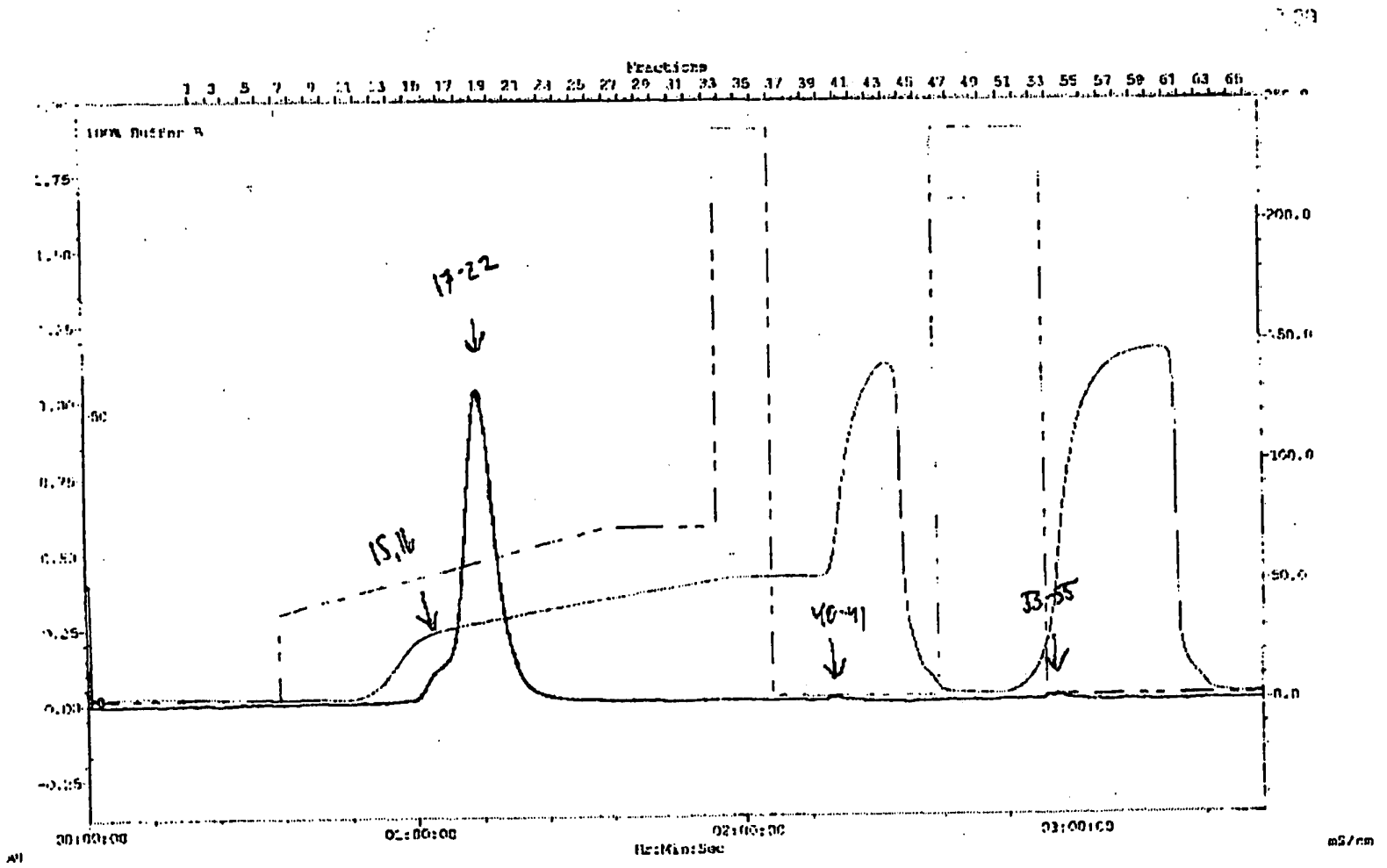


Figure 1

1/4

Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10mM + 1MNaCl, pH 8.5

Flow Rate: 1 ml/min

Gradient:

Chart Speed:

Fraction Size: 3 ml

Run Description: HaAL fallt enligt caseinprotokoll

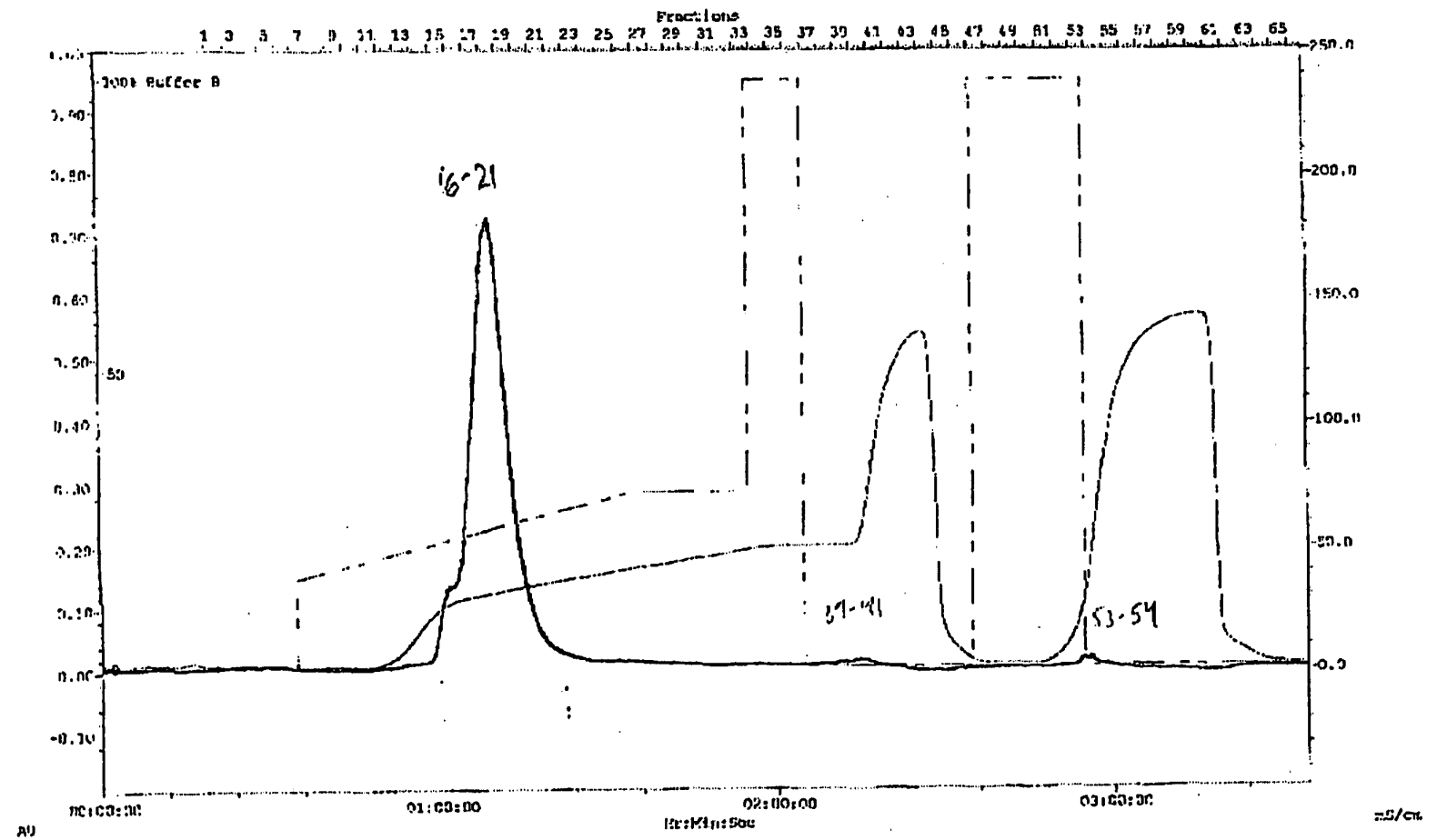


Figure 2

2/4

Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10mM + 1MNaCl, pH 8.5

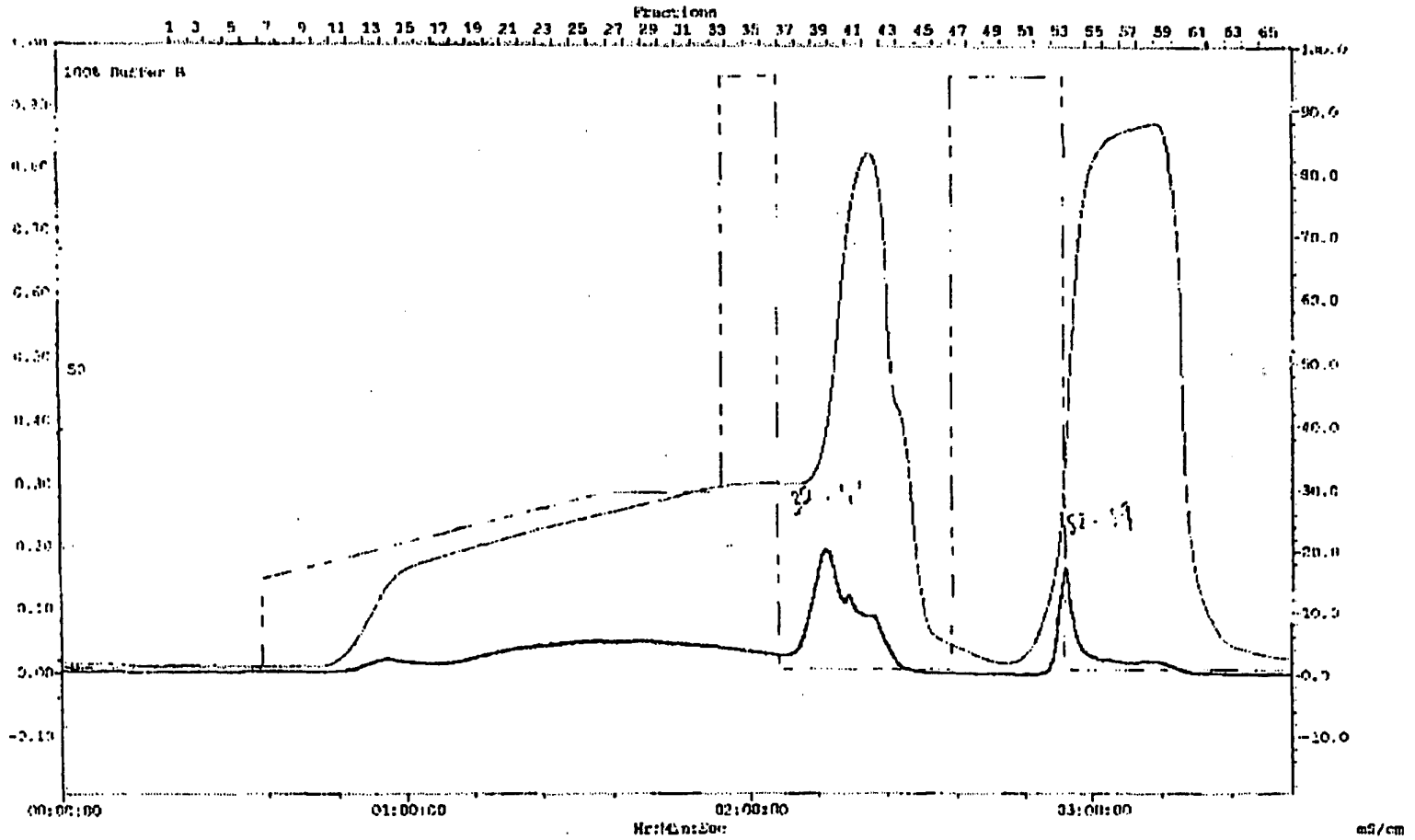
Flow Rate: 1 ml/min

Gradient:

Chart Speed:

Fraction Size: 3 ml

Run Description: Hum aLA +lipid caseinfallning



Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10mM + 1MNaCl, pH 8.5

Operator: Ankl

Flow Rate: 1 ml/min

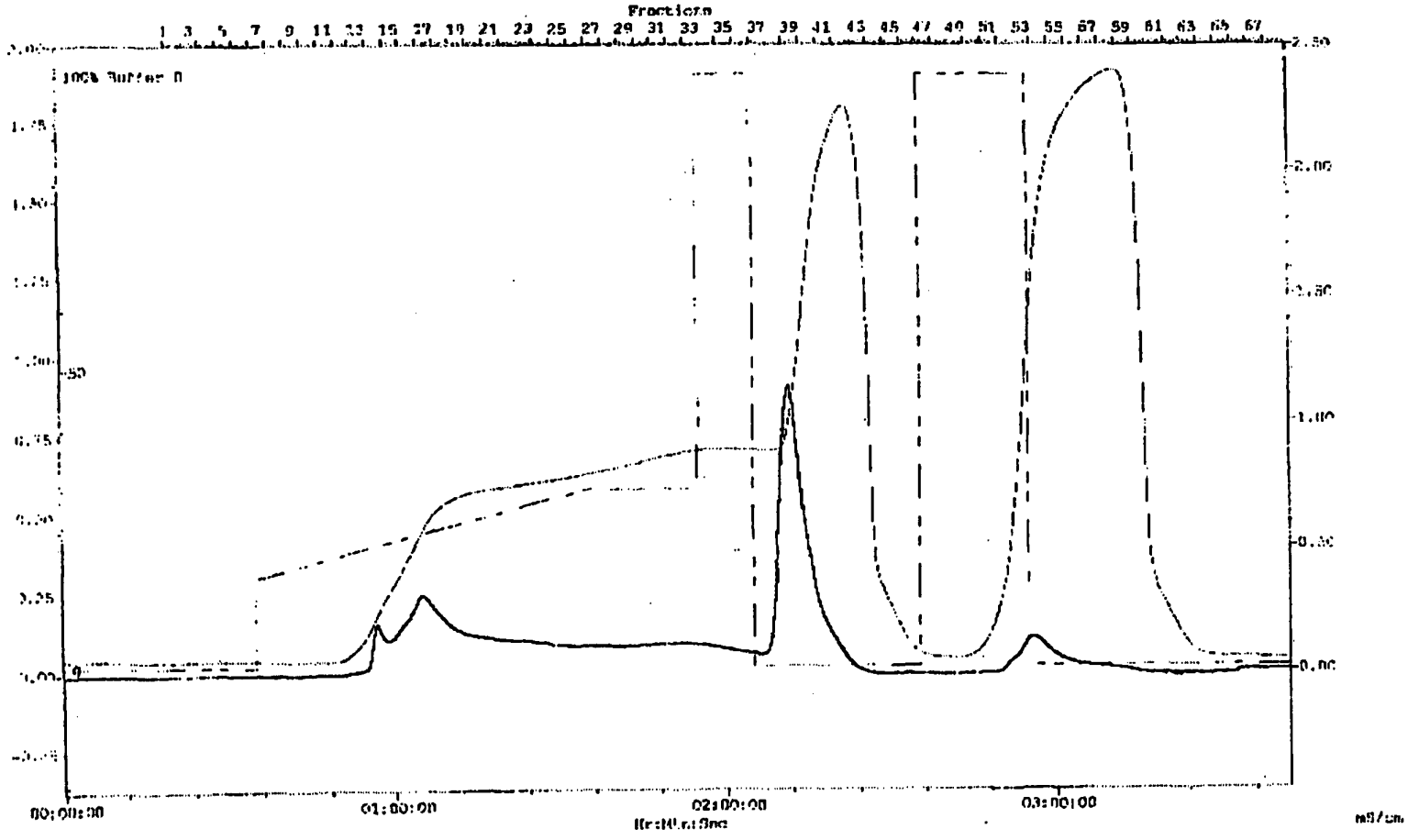
Gradient:

Chart Speed:

Fraction Size: 3 ml

Run Description: obeh Hala tranad pelare

Figure 3



Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10mM + 1MNaCl, pH 8.5

Operator: Anki

Flow Rate: 1 ml/min

Gradient:

Chart Speed:

Fraction Size: 3 ml

Run Description: bovin aLA (ca 10mg)

FIGURE 4